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Original Article

Effects of Colcemid on Mitotic Index and Metaphase Quality for Cytogenetic Dosimetry

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Abstract: Cytogenetic dosimetry plays an important role in assessing individual exposure to ionizing radiation, especially through analysis of dicentric chromosomes (DC). For DC assessment, peripheral blood lymphocytes are mitogen-stimulated and cultured with Colcemid added to arrest cells at metaphase for chromosome analysis. This study evaluated how Colcemid concentration and the timing of its addition to the culture affected the mitotic index (MI), the frequency of first-division metaphases (FM1), and the quality of chromosomal contraction for microscopic analysis. Peripheral blood samples from two volunteers were irradiated with 6 Gy and cultured for 72 hours with different concentrations of Colcemid (0.05 and 0.10 µg/mL), added at 0, 24, and 45 h. In irradiated samples, early addition (0 h) of 0.05 µg/mL Colcemid yielded the most favorable balance, with higher MI values (≈ 9 %) than samples treated with 0.10 µg/mL, while maintaining FM1 > 90 % and adequate chromosomal contraction. An explicit MI × FM1 trade-off emerged: the higher concentration maximized the first-division fraction (FM1 ≥ 99 %), the endpoint that directly conditions dicentric scoring, but reduced the MI of irradiated cultures (6.8 % vs 9.0 %), whereas the lower concentration preserved mitotic progression at the cost of a modestly lower FM1 (≈ 94 %). Delayed addition (24 and 45 h) improved no endpoint and further reduced the first-division yield. These findings show that Colcemid optimization requires balancing mitotic yield against first-division predominance, given the combined cytotoxic effects of Colcemid and ionizing radiation, which directly influence the accuracy and efficiency of dosimetric assessment across different exposure situations.

Keywords: cytogenetic dosimetry, colcemid, mitotic index, radiation exposure.

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Efeitos do Colcemid sobre o índice mitótico e a qualidade das metáfases na dosimetria citogenética

Resumo: A dosimetria citogenética desempenha um papel importante na avaliação da exposição individual à radiação ionizante, especialmente por meio da análise de cromossomos dicêntricos (CD). Para a contagem de CD, linfócitos do sangue periférico são estimulados com mitógenos e cultivados, com a adição de Colcemid para induzir a parada do ciclo celular na ressubmissão metáfase, permitindo a análise cromossômica. Este estudo avaliou os efeitos da concentração de Colcemid e do tempo de adição à cultura sobre o índice mitótico (IM), a frequência de metáfases na primeira divisão (FM1) e a qualidade da contração cromossômica para análise microscópica. Amostras de sangue periférico de dois voluntários foram irradiadas com 6 Gy e cultivadas por 72 horas em diferentes concentrações de Colcemid (0,05 e 0,10 µg/mL), adicionadas às 0, 24 e 45 h. Nas amostras irradiadas, a adição precoce (0 h) de 0,05 µg/mL de Colcemid proporcionou o melhor equilíbrio, com valores de IM mais elevados (≈ 9 %) em comparação às amostras tratadas com 0,10 µg/mL, mantendo FM1 > 90 % e uma contração cromossômica adequada. Emergiu um trade-off explícito entre IM e FM1: a maior concentração maximizou a fração de primeira divisão (FM1 ≥ 99 %), o desfecho que condiciona diretamente a contagem de dicêntricos, mas reduziu o IM das culturas irradiadas (6,8 % vs 9,0 %), enquanto a menor concentração preservou a progressão mitótica ao custo de FM1 modestamente inferior (≈ 94 %). A adição tardia (24 e 45 h) não melhorou nenhum desfecho e reduziu ainda mais o rendimento de primeira divisão. Esses achados demonstram que a otimização do Colcemid exige equilibrar o rendimento mitótico e a predominância de primeira divisão, no contexto dos efeitos citotóxicos combinados do Colcemid e da radiação ionizante, que influenciam diretamente a precisão e a eficiência da avaliação dosimétrica em diferentes cenários de exposição.

Palavras-chave: dosimetria citogenética, colcemid, índice mitótico, exposição à radiação.

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1. INTRODUCTION

Cytogenetic dosimetry, also called biodosimetry, is an important tool for assessing individual exposure to ionizing radiation in occupational, environmental, and clinical contexts. Among available methods, the dicentric chromosome assay (DCA) is considered the gold standard for biodosimetry because of its high specificity and reliability in quantifying radiation-induced chromosomal damage. DCA estimates absorbed dose from the frequency of unstable chromosomal aberrations, particularly dicentric chromosomes, in peripheral blood lymphocytes [1-3].

To perform DCA, lymphocytes are stimulated with mitogens to enter cell division and cultured *in vitro* for approximately 48 hours, although the time may vary depending on laboratory conditions and protocols [1]. During cell cultivation, Colcemid, an antimicrotubule agent that inhibits mitotic spindle formation, is added to the medium to arrest cell division at metaphase, when chromosomes are highly condensed and clearly defined [1,2]. This period is crucial for maximizing the proportion of cells in the first mitotic division (M1), as these aberrations tend to be lost in subsequent divisions.

The fluorescence plus Giemsa (FPG) technique often distinguishes metaphase cells in the first division (M1) from those in the second division (M2) by differentially staining sister chromatids. However, the FPG approach has limitations, such as slide degradation and variations in staining intensity, which can affect analytical accuracy. Thus, strategies that favor M1 predominance, such as adjusting the concentration and timing of Colcemid addition, have been proposed to simplify and optimize the protocol, reducing reliance on FPG [1,4].

However, Colcemid use presents challenges. Its toxicity may affect the mitotic index and the frequency of first-division metaphases, depending on the concentration and timing of addition to the cell culture, and may impair chromosomal contraction, compromising the

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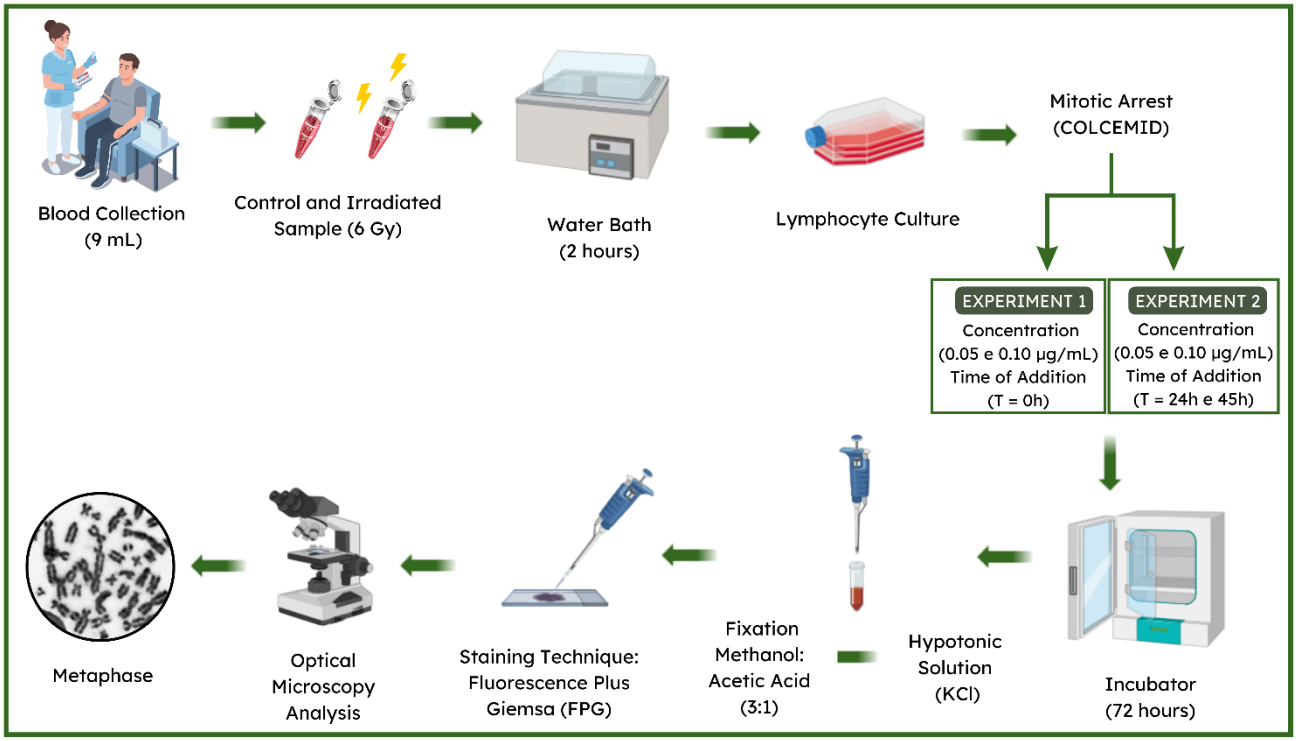
accuracy of absorbed dose estimates [5-8]. For this reason, optimizing these variables is crucial to ensure reproducible and reliable results.

In this context, this study investigated the influence of Colcemid concentration and its addition time on the mitotic index (MI), the frequency of first-division metaphases (FM1), and the quality of chromosome spreads, to establish optimal conditions that maximize the yield of well-defined metaphases and minimize interference with chromosomal analysis.

2. MATERIALS AND METHODS

Figure 1 summarizes the experimental workflow of this study.

Figure 1: Experimental workflow of this study, outlining the key steps from sample collection and irradiation through to cytogenetic processing and microscopic analysis.



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2.1. Collection and Irradiation of Samples

After obtaining written informed consent from each donor (Ethics Committee registration number: 6.337.895), 9 mL of peripheral blood was collected from two volunteers. Each blood sample was divided into two groups: control (non-irradiated, NIR) and irradiated (6 Gy). The irradiation process was performed using a Gammacell irradiator at the Department of Nuclear Energy, Federal University of Pernambuco, equipped with a ^{60}Co source and operating at an average dose rate of 1.0335 kGy/h. Immediately after irradiation, the samples were incubated in a 37 °C water bath for 2 hours to allow DNA repair.

2.2. Lymphocyte Culture

Lymphocytes were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 2 % phytohemagglutinin, and 30 µM bromodeoxyuridine (BrdU), following the methodology recommended by the International Atomic Energy Agency (IAEA) [1].

Colcemid at 0.05 and 0.10 µg/mL was added under two experimental conditions:

- **Experiment 1 (E1)**: at the beginning of the culture (t = 0);
- **Experiment 2 (E2)**: 24 h and 45 h after culture initiation.

The cultures were maintained in a humidified 5 % CO_2 incubator at 37 °C. After 72 h of culture, cell suspensions were centrifuged at 300 g for 5 min, treated with a hypotonic solution of 75 mM potassium chloride at 37 °C for 7 min in a water bath, and fixed with a methanol-acetic acid solution (3:1). After successive washes with the fixative, the samples were distributed on slides and stained by the fluorescent plus Giemsa (FPG) technique, following the methodology of Ferreira-Lucena et al. [9].

2.3. Mitotic Index, Frequency of First-Division Metaphases, and Quality of Metaphases

Samples were analyzed by conventional light microscopy at the Laboratory of Applied Modeling and Biodosimetry (LAMBDA), Department of Nuclear Energy, Federal University

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of Pernambuco (UFPE), employing IAEA scoring criteria to quantify lymphocyte metaphases [1]. To ensure uniform sampling, each slide was divided into four quadrants. A total of 1,000 cells were counted per slide (250 cells per quadrant). The mitotic index was calculated as the ratio of total metaphases to the total number of analyzed cells, according to Equation 1.

$$Mitotic\ Index = \frac{Metaphases \times 100}{Total\ Cells} \qquad (1)$$

The frequency of metaphases in the first division was determined by the ratio between the number of metaphases in the first division and the total number of metaphases observed, according to Equation 2.

$$Frequency\ of\ M1 = \frac{(Metaphases\ in\ first\ division) \times 100}{Total\ Metaphases} \qquad (2)$$

To assess chromosome quality, first-division metaphases were evaluated for qualitative morphological parameters, specifically chromosomal contraction and centromere visibility.

2.4. Statistical Analysis of the Results

For each endpoint (mitotic index, MI; frequency of first-division metaphases, FM1; frequency of scorable metaphases, FSCM), the slide was defined as the experimental unit. In experiment 1 (E1), 24 slides (six per condition; 1,000 cells scored per slide; 250 per quadrant; four quadrants per slide) were analyzed across four conditions (non-irradiated (Control) and irradiated (6 Gy) × Colcemid concentration: 0.05 and 0.1 μg/mL), with Colcemid added at culture initiation. In experiment 2 (E2) (delayed Colcemid addition at 24 h and 45 h across the same four conditions; n = 3 per condition), 24 additional slides were analyzed. Cells and metaphases within a slide are subsamples and were not treated as independent observations; all hypothesis tests were therefore performed on slide-level values, with N referring to the number of slides. Donor was treated as a blocking factor (n = 2). Normality of slide-level values was assessed with the Shapiro-Wilk test. Two-group comparisons used Student's t-test, with Welch's correction when variances were unequal, and the Mann-Whitney U test when normality was rejected. Comparisons involving more than two groups, or two factors (dose ×

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Colcemid concentration), used the Kruskal-Wallis test and two-way ANOVA on slide-level values, as appropriate. Given the limited number of donors, results are interpreted as protocol-level optimization rather than population-level inference; effect sizes (Cohen's d) with 95 % confidence intervals are reported alongside exact p-values. Significance was set at $p \leq 0.05$.

3. RESULTS AND DISCUSSIONS

3.1. Mitotic Index and Frequency of Metaphases

Table 1 presents the mitotic index (MI) results for non-irradiated controls and 6 Gy-irradiated samples at different Colcemid concentrations added at culture initiation (t = 0 h).

Table 1: Mitotic index (MI) in Experiment 1 for non-irradiated (NIR) control and 6 Gy-irradiated samples at different Colcemid concentrations (time of addition = 0 h).

SAMPLES	COLCEMID (µg/mL)	MITOTIC INDEX (Mean ± SD (%))	SHAPIRO-WILK (p-value)*
Control (NIR)	0.05	14.47 ± 1.82	0.432^{ns}
	0.10	19.60 ± 4.03	0.284^{ns}
6 Gy-Irradiated	0.05	8.98 ± 1.77	0.328^{ns}
	0.10	6.77 ± 1.65	0.578^{ns}

*[ns] not significant at $\alpha > 0.05$

In Experiment 1 (E1; **Table 1**), irradiation with 6 Gy reduced the MI at both Colcemid concentrations. At 0.05 µg/mL, MI decreased from (14.47 ± 1.82) % in non-irradiated cultures to (8.98 ± 1.77) % in irradiated cultures (Student's t-test: $p = 3.5 \times 10^{-4}$, d = 3.06; Mann-Whitney test: p = 0.0082, r = 0.76). At 0.1 µg/mL, MI decreased from (19.60 ± 4.03) % to (6.77 ± 1.65) % (Student's t-test: $p = 2.8 \times 10^{-5}$, d = 4.17; Mann-Whitney test: p = 0.0050, r = 0.81).

When data from both concentrations were pooled, the influence of irradiation remained highly significant for mitotic index ($p = 4.2 \times 10^{-7}$, d = 2.89). On the other hand, the effect of Colcemid concentration differed according to irradiation status: in non-irradiated cultures, MI was higher at 0.1 than at 0.05 µg/mL ((19.60 ± 4.03) % vs (14.47 ± 1.82) %; p = 0.017, d

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= −1.64), whereas in irradiated cultures, the reverse pattern was observed, with a lower MI at 0.1 than at 0.05 μg/mL ((6.77 ± 1.65) % vs (8.98 ± 1.77) %; p = 0.049, d = 1.30).

For Experiment 2, **Table 2** presents descriptive statistics and Shapiro-Wilk test results for the mitotic index at the tested Colcemid concentrations at two addition times, namely 24 and 45 h.

Table 2: Descriptive statistics and Shapiro-Wilk normality test results for the mitotic index in Experiment 2, evaluated as a function of Colcemid concentration and time of addition.

Irradiation	Addition Time (h)	COLCEMID (μg/mL)	Mitotic Index (%)		Shapiro-Wilk (p-value)*
			Median	IQR	
Control (non-irradiated)	24	0.05	13.5	(12.30 - 13.70)	0.253
		0.10	9.2	(8.50 - 10.25)	0.780
	45	0.05	10.1	(10.05 - 11.55)	0.056
		0.10	8.2	(7.80 - 8.60)	1.000
6 Gy	24	0.05	6.0	(4.90 - 6.45)	0.546
		0.10	2.6	(2.55 - 2.95)	0.220
	45	0.05	3.2	(3.05 - 3.20)	0.001[s]
		0.10	4.0	(2.95 - 4.05)	0.077

IQR - Interquartile Range; *[s] significant at α ≤ 0.05

In this case, the time of Colcemid addition (24 h vs. 45 h) was not associated with a significant difference in MI (p = 0.444; d = 0.318). In contrast, radiation dose remained the dominant factor strongly affecting MI (two-way ANOVA: F = 79.28, p < 0.0001, $\eta p^2 = 0.799$).

MI also differed across the eight experimental conditions (Kruskal-Wallis: H = 20.61, df = 7, p = 0.004, $\varepsilon^2 = 0.896$). Mean MI ranged from 2.8 % to 5.6 % in irradiated cultures, compared with 8.2 % to 12.8 % in controls. The yield of first-division metaphases per cell counted (MI1 = MI × FM1) was also lower after irradiation (2.5 % - 4.9 % at 6 Gy vs. 6.3 % - 9.1 % at 0 Gy), consistent with the combined effects of radiation-induced cytotoxicity and delayed mitotic arrest. Because replication was limited (n = 3) and some data departed from normality, parametric and nonparametric tests were performed in parallel, which agreed in all cases.

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The analysis of both experiments (E1 and E2) revealed consistent patterns, confirming a clear correlation between radiation exposure and a decreased Mitotic Index (MI). This reduction in cell proliferation in irradiated samples can be attributed to the activation of DNA damage response mechanisms at cell cycle checkpoints. Under these conditions, cells may arrest the cycle to undergo repair – resuming division upon successful correction – or trigger apoptosis if the damage is irreparable [10-13].

Evaluation of MI values indicates that early addition of Colcemid (0 h) at 0.05 µg/mL and 0.10 µg/mL is optimal for promoting cell proliferation, even at high radiation doses (6 Gy). Using a much lower concentration (0.01 µg/mL) of Colcemid, Ferreira-Lucena et al. [9] reported MI values in the range of 5-6 % when cells were irradiated with 6 Gy, whereas the present study observed 9 % at 0.05 µg/mL and 7 % at 0.10 µg/mL under similar conditions (**Table 1**). Kanda et al. [5] also reported a decrease in MI when Colcemid concentrations were below 0.05 µg/mL. Moreover, adding Colcemid at later times (24 h and 45 h) in E2 also resulted in lower MI values, ranging from 3 % to 6 % (**Table 2**).

These findings are consistent with Da Matta et al. [14], who emphasize the importance of Colcemid concentration and timing for metaphase yield, which can substantially affect chromosome scoring. For example, a too-low concentration of Colcemid can lead to poor MI, even when added at the beginning of culture [8]. Furthermore, prolonged Colcemid exposure can induce excessive chromosomal condensation, impairing cytogenetic analysis [15].

When analyzing the influence of Colcemid concentration, MI was higher in the E1 control samples at 0.10 µg/mL (19.6 %) than at 0.05 µg/mL (14.5%). However, in irradiated samples, the lower concentration provided a higher MI (8.98 % vs. 6.8 %; **Table 1**). This divergent behavior suggests that, in cells without physical stress (radiation exposure), the highest Colcemid concentration may favor mitotic blockade. In irradiated cells, however, the interaction between Colcemid toxicity and radiation further compromises progression into metaphase, requiring additional time for DNA repair and cell-cycle progression [9]. Strict control of these experimental variables is critical to optimize mitotic yield.

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Using reduced concentrations of Colcemid, as in E1, minimizes adverse effects on cell proliferation and favors the detection of metaphases representative of post-irradiation cellular conditions, reinforcing the methodology's effectiveness and aligning with previous studies [5,16].

3.2. Frequency of First-Division Metaphases

Table 3 presents descriptive statistics for first-division metaphase frequency at the tested Colcemid concentrations and irradiation doses in Experiment 1 (E1)**.**

Table 3: Means, standard deviations, and median (IQR) of first-division metaphase frequency by Colcemid concentration and irradiation dose in Experiment 1.

Irradiation	Colcemid (µg/mL)	Mean ± SD (%)	Median (IQR) (%)	Shapiro-Wilk (*p*-valor)*
Control (non-irradiated)	**0.05**	95 ± 1.7	95 (94 - 96)	0.252^{ns}
	0.10	99 ± 1.2	100 (99 - 100)	0.003^{s}
6 Gy	**0.05**	93.8 ± 2.04	94 (92 - 96)	0.393^{ns}
	0.10	99.7 ± 0.82	100 (100 - 100)	0.001^{s}

IQR - Interquartile Range; *[s] significant at $\alpha \leq 0.05$

In this Experiment, in which Colcemid was added at culture initiation (0 h), FM1 remained high across all conditions. Mean values ranged from 93.8 % to 99.7 %, with little variability (**Table 3**).

Among the outcomes assessed, FM1 showed the strongest association with Colcemid concentration (two-way ANOVA: $F = 61.27$, $p = 1.6 \times 10^{-7}$, $\eta p^2 = 0.754$). At 0.1 µg/mL, at least 99 % of classified metaphases were in the first division: (99.32 ± 1.20) % at 0 Gy and (99.61 ± 0.96) % at 6 Gy. At 0.05 µg/mL, the corresponding values were approximately 94%: (94.92 ± 1.62) % at 0 Gy and (93.97 ± 2.21) % at 6 Gy.

A comparison pooling the radiation dose groups confirmed this difference ($p = 6.3 \times 10^{-8}$, $d = -3.25$). Neither radiation dose ($p = 0.609$; Mann-Whitney test: $U = 69.0$, $p = 0.882$) nor the interaction between dose and concentration ($p = 0.343$) was statistically significant.

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These results indicate that radiation exposure was not associated with FM1 when Colcemid was added at the start of culture.

Table 4 presents the median frequencies of M1 and M2 metaphases across Colcemid concentrations, addition times, and irradiation doses in Experiment 2 (E2), along with the Shapiro-Wilk test results.

Table 4: Median (IQR) of M1 and M2 metaphase frequency by Colcemid concentration, addition time, and irradiation dose in Experiment 2, with Shapiro-Wilk test results.

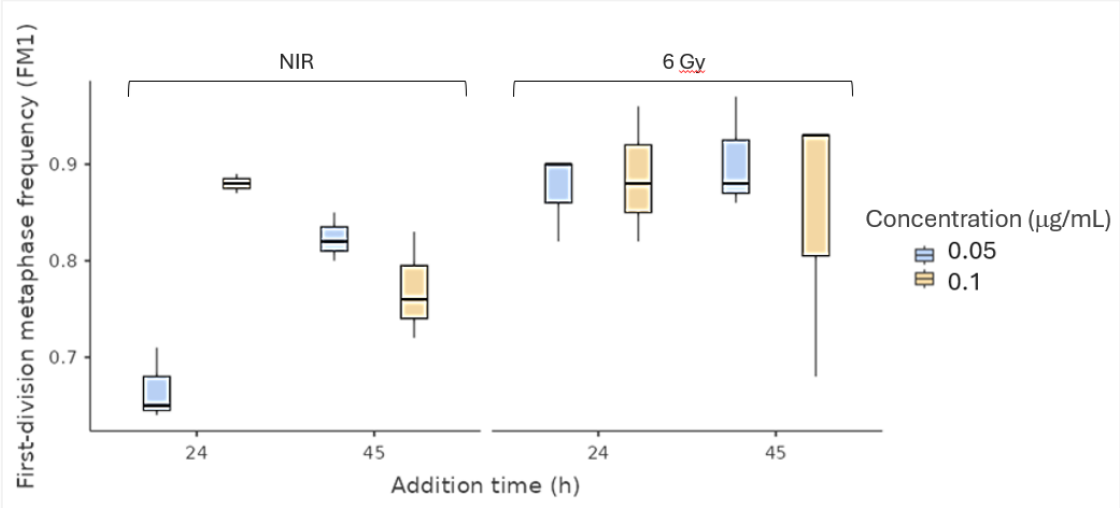
Irradiation	Addition Time (h)	Colcemid (µg/mL)	M1(%)	M2(%)	Shapiro-Wilk
			Median (IQR)	Median	(*p-value*)*
Control (non-irradiated)	24	0.05	65.0 (64.5 - 68.0)	35.0	0.253^{ns}
		0.10	88.0 (87.5 - 88.5)	12.0	1.000^{ns}
	45	0.05	82.0 (81.0 - 83.5)	18.0	0.780^{ns}
		0.10	76.0 (74.0 - 79.5)	24.0	0.702^{ns}
6 Gy	24	0.05	90.0 (86.0 - 90.0)	10.0	0.001^{s}
		0.10	88.0 (85.0 - 92.0)	12.0	0.843^{ns}
	45	0.05	88.0 (87.0 - 92.5)	12.0	0.328^{ns}
		0.10	93.0 (80.5 - 93.0)	7.0	0.001^{s}

IQR - Interquartile Range; *[s] significant at $\alpha \leq 0.05$

With Colcemid added at either 24 or 45 h, FM1 values ranged from 65 % to 97 % across slides. These values were consistently below the near-complete predominance of first-division metaphases observed in E1 (FM1 > 0.93; **Figure 2**), indicating a greater contribution of second- and third-division metaphases in E2.

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Figure 2: Dose-dependent variation in FM1 (%) under delayed Colcemid addition (24-45 h) (E2).



Radiation dose had a significant effect on FM1 (two-way ANOVA: $F = 6.81$, $p = 0.017$, $\eta p^2 = 0.254$), whereas the pooled effect of Colcemid addition time and concentration was not significant ($p = 0.818$, $d = 0.095$).

A significant interaction between addition time and concentration was observed ($F = 5.46$, $p = 0.030$, $\eta p^2 = 0.214$). Among non-irradiated cells treated with 0.05 µg/mL Colcemid, FM1 was lower when Colcemid was added at 24 h than at 45 h ((66.7 ± 3.8)% vs. (82.3 ± 2.5)%; $p = 0.004$, $d = 4.87$). At 0.1 µg/mL, the pattern was reversed ((88.0 ± 1.0) % at 24 h vs. (77.0 ± 5.6) % at 45 h; $p = 0.028$, $d = 2.75$). Again, because only three slides were analyzed per condition, this interaction should be interpreted cautiously. The Kruskal-Wallis test also found no statistically significant differences in FM1 across the eight conditions ($p = 0.079$).

This behavior can be explained by radiation-induced cell cycle delay. Ionizing radiation activates DNA damage checkpoints, slowing cell-cycle progression and causing cells to accumulate in the first division. In contrast, non-irradiated cells progress more rapidly to subsequent divisions (M2), reducing FM1. These findings are consistent with previous studies describing radiation-induced mitotic delay and cell cycle arrest [16-19].

Notably, in E2, FM1 medians ranged from approximately 65 % to 93 %, substantially lower than those observed in E1 (> 94 %) (**Figure 2**). This confirms that the timing of

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Colcemid addition is the dominant factor controlling FM1, overriding concentration effects under delayed addition conditions.

Overall, the comparison between the two experiments clearly demonstrates that: (a) early Colcemid addition (0 h) ensures consistently high FM1 (> 90 %), enabling efficient capture of metaphase cells, even in control samples; (b) delayed addition (24-45 h) of Colcemid leads to reduced FM1 and increased variability, favoring the occurrence of second-division metaphases; c) under delayed conditions, radiation dose becomes the primary determinant of FM1.

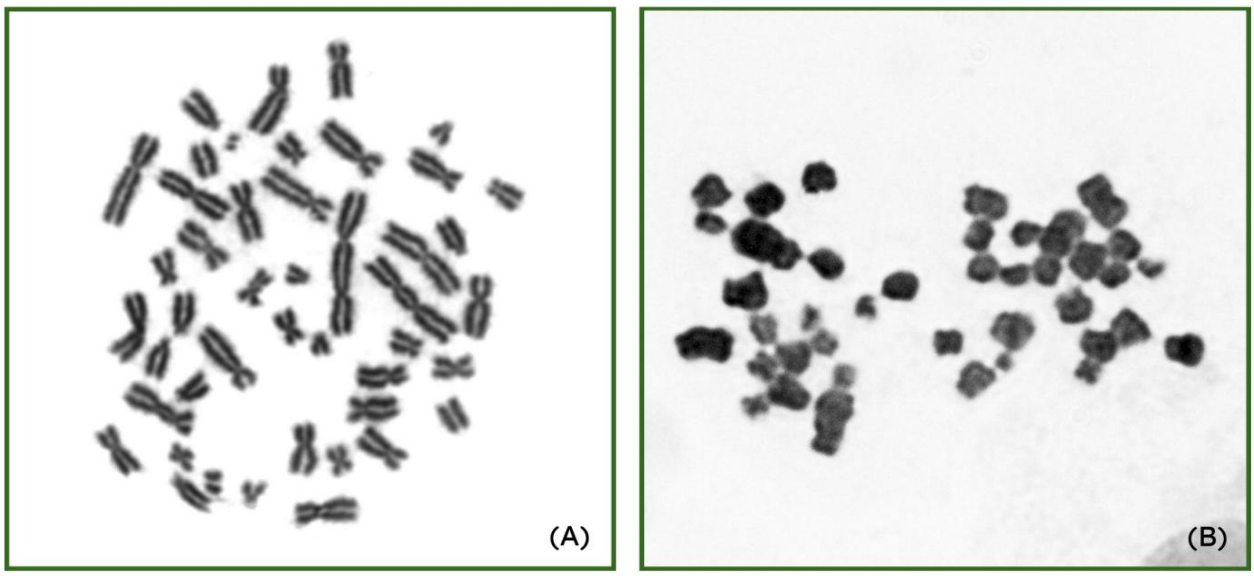
Methodologically, achieving high FM1 is critical for cytogenetic dosimetry, as unstable chromosomal aberrations such as dicentrics are progressively lost in subsequent divisions. Therefore, protocols that maximize FM1 improve both analytical efficiency and dosimetric accuracy. Furthermore, when FM1 is around or above 95%, the need for differential staining techniques, such as FPG, is minimized, reducing analytical complexity and potential sources of error [1,20,21].

3.3. Quality of Metaphases

First-division metaphases from Experiment 1 were classified as satisfactory or unsatisfactory based on the degree of chromosomal contraction and centromere visibility (**Figure 3**). This analysis allowed the evaluation of the influence of Colcemid added at the beginning of the culture, a condition in which its toxic effects may be more evident.

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Figure 3: Representative micrographs of first-division metaphase classification. (A) Satisfactory metaphase exhibiting optimal chromosomal contraction and clear centromere visibility. (B) Unsatisfactory metaphase showing poor chromosome morphology or excessive contraction.



The frequency of satisfactorily contracted metaphases (FSCM) in Experiment 1 (E1) ranged from 57.5 % to 66.3 %, with mean values centered around 60 % across all experimental conditions (**Table 5**). These results indicate a moderate but consistent level of chromosomal quality suitable for cytogenetic analysis.

Table 5: Means and standard deviations of the frequency of satisfactorily contracted metaphases (FSCM) according to irradiation dose and Colcemid concentration added at 0 h.

Irradiation	Colcemid (µg/mL)	FSCM Mean± SD (%)	Shapiro-Wilk (*p-value*)*
Control (Non-Irradiated)	**0.05**	66.3 ± 7.5	0.401^{ns}
	0.10	63.3 ± 8.6	0.125^{ns}
6 Gy	**0.05**	63.1 ± 6.9	0.059^{ns}
	0.10	57.5 ± 7.4	0.647^{ns}

***[ns] not significant at α > 0,05

The Shapiro-Wilk test confirmed normal distribution of the slide-level FSCM values in all groups ($p > 0.05$), allowing parametric analysis: two-way ANOVA revealed no significant effect of dose ($F = 2.076$; $p = 0.165$), of Colcemid concentration ($F = 1.903$; $p = 0.183$), or of their interaction ($F = 0.181$; $p = 0.675$).

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Although irradiated samples at 0.10 µg/mL showed a slight reduction in FSCM (mean = 57.5 %), this decrease was not statistically significant. This suggests that chromosomal morphology remains preserved even under genotoxic stress, which is essential for the reliable identification of chromosomal aberrations. Given six slides per condition, absence of significance should not be interpreted as absence of an effect, and the descriptive trend towards the lowest FSCM in irradiated cultures treated with 0.1 µg/mL warrants monitoring in follow-up experiments with additional donors. Operationally, given the FSCM (~ 60 %) and the mitotic index, we obtained approximately 50-60 analyzable metaphases per slide. This meets the IAEA recommendations for initial triage in radiological emergencies, which require the analysis of approximately 50 metaphases per individual [1], and enables analysis from a single slide, thus optimizing laboratory resources

As shown in Table 5, 0.05 µg/mL Colcemid added at 0 h balanced adequate chromosomal condensation quality (with FSCM comparable to 0.10 µg/mL), reduced cytotoxicity (as indicated by a higher mitotic index), and sufficient metaphase yield for efficient analysis.

These findings reinforce the critical role of Colcemid in chromosomal quality and cytotoxic stress, particularly in irradiated samples. Excessive Colcemid treatment may induce over-condensation or structural distortion of chromosomes [14,15]. Optimizing Colcemid use requires balancing concentration and exposure time to maximize the quantity and quality of metaphase cells for each culture setup and objective. For example, Da Matta et al. [14] observed that, for 48 h cultures, 0.05 µg/mL of Colcemid added at 0 h produced chromosomes with approximately half the average length compared with cultures where 0.5 µg/mL Colcemid was added at 45 h. Although we have not analyzed the effects on chromosome length, adding Colcemid at 0 h for a 72-h culture had the highest values of Mitotic Index (MI) and frequency of first-division metaphases (FM1), as discussed in previous topics, and yielded about 57.5-66.3 % of analyzable metaphases. Prolonged culture

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(72 h) can be particularly effective in overcoming low MI when lymphocytes are exposed to high radiation doses (> 5 Gy) [9] and interpreting partial-body exposures [16].

Lastly, besides chromosome condensation and MI, Colcemid concentration also influences chromosome spreading and morphology [7]. Thus, understanding the effects of Colcemid on lymphocytes is fundamental when deciding which combination of concentration and exposure time maximizes results.

4. CONCLUSIONS

The concentration of 0.05 μg/mL of Colcemid, added at the beginning of the culture, proved to be the most appropriate condition for this experimental protocol. This strategy achieved a satisfactory mitotic index (MI), even after exposure to high radiation doses, and a first-division metaphase (FM1) frequency greater than 90 %. The combination of lower concentration and addition at 0 h also mitigated negative effects, such as decreases in MI and FM1, observed under other tested conditions. Moreover, approximately 60% of the metaphases analyzed showed satisfactory chromosomal condensation, enabling clear visualization of the chromosomes. The average number of metaphases per slide met the IAEA recommendations for initial screening in a radiological emergency, enabling analysis from a single slide and optimizing laboratory resources.

This work demonstrated that the optimized methodology is efficient, simple, and applicable across diverse radiation-exposure contexts, making it a viable approach for cytogenetic screening and dose estimation with precision and reliability.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the results of this study are available within the article. Derived data supporting the findings of this study that are not found in the article may be requested from the corresponding author.

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